JC05 Rec'd PCT/PTO 25 JUL 2001

Express Mail Label No.: EL 920 877 057 US

FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: CIBT-P01-097

U S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/890053

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/00847	3 February 2000	4 February 1999

TITLE OF INVENTION: Pharmaceutical Composition of Hydrophobically Modified Hedgehog Proteins and Their Use

APPLICANT(S) FOR DO/EO/US: PAPADIMITRIOU, Apollon and LANG, Kurt

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **unexecuted**
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification..
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

531 Rec'd PCT/PTC 25 JUL 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
NEW 09/890053	PCT/EP00/00847	CIBT-P01-097

21. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	12 - 20 =	0	x $18.00	$ 0.00
Independent claims	3 - 3 =	0	x $80.00	$ 0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 270.00
TOTAL OF ABOVE CALCULATIONS =				$ 1,130.00
[X] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ 565.00
SUBTOTAL =				$ 565.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 565.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$ 565.00
			Amount to be refunded:	$
			charged:	$ 565.00

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 18-1945 in the amount of $ 565.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1945. A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Ropes & Gray
Patent Group
One International Place
Boston, Massachusetts 02110

SIGNATURE

David P. Halstead
NAME

44,735
REGISTRATION NUMBER

Pharmaceutical composition of hydrophobically modified hedgehog proteins and their use

The invention concerns a pharmaceutical composition of hydrophobically modified hedgehog proteins and their use, in particular for the local release of these proteins on bones or cartilage.

Hedgehog (hh) proteins are understood as a family of secreted signal proteins which are responsible for the formation of numerous structures in embryogenesis (J.C. Smith, Cell 76 (1994) 193 - 196, N. Perrimon, Cell 80 (1995) 517 - 520, C. Chiang et al., Nature 83 (1996) 407, M.J. Bitgood et al., Curr. Biol. 6 (1996) 296, A. Vortkamp et al., Science 273 (1996) 613, C.J. Lai et al., Development 121 (1995) 2349). During its biosynthesis a 20 kD N-terminal domain and a 25 kD C-terminal domain are obtained after cleavage of the signal sequence and autocatalytic cleavage. In its natural form the N-terminal domain is modified with cholesterol (J.A. Porter et al., Science 274 (1996) 255 - 259). In higher life-forms the hh family is composed of at least three members namely sonic, indian and desert hh (shh, Ihh, Dhh; M. Fietz et al., Development (Suppl.) (1994) 43 - 51). Differences in the activity of hedgehog proteins that were produced recombinantly were observed after production in prokaryotes and eukaryotes (M. Hynes et al., Neuron 15 (1995) 35 - 44 and T. Nakamura et al., Biochem. Biophys. Res. Comm. 237 (1997) 465 - 469).

Hynes et al. compare the activity of hh in the supernatant of transformed human embryonic kidney 293 cells (eukaryotic hh) with hh produced from E. coli and find a four-fold higher activity of hh from the supernatants of the kidney cell line. The reason for this increased activity has been discussed to be a potential additional accessory factor which is only expressed in eukaryotic cells, a post-translational modification, a different N-terminus since the hh isolated from E. coli contains 50 % of a hh form which carries two additional N-terminal amino acids (Gly-Ser) or is shortened by 5 - 6 amino acids, or a higher state of aggregation (e.g. by binding to nickel agarose beads).

Nakamura et al. compare the activity of shh in the supernatant of transformed chicken embryo fibroblasts with an shh fusion protein isolated from E. coli which still has an N-terminal polyhistidine part. The shh in the supernatant of the fibroblasts has a seven-fold higher activity than the purified E. coli protein with regard to stimulation of alkaline phosphatase (AP) in C3H10T ½ cells. The increased activity has been postulated to be due molecules such as for example bone morphogenetic proteins (BMPs) which are only present in the supernatant of eukaryotic cells and cause the stronger induction of AP.

Kinto et al., FEBS Letters, 404 (1997) 319 - 323 describe that fibroblasts which secrete hh induce ectopic bone formation in an i.m. implantation on collagen. Thus hedgehog proteins have an osteoinductive activity.

A process for the production of delivery systems for proteins with a delayed release using alginate is known from WO 90/08551. A two-phase system is formed in which the first phase contains a high concentration of the protein (saturated solution) and the second phase contains alginate. However, such a phase separation is difficult and complicated to carry out when producing pharmaceutical compositions in large amounts.

A pulsatile release of dextran from calcium alginate complexes is known from Kikuchi, A. et al., J. Controlled Release 47 (1997) 21-29. However, the coupling of hedgehog proteins to such complexes is not described by Kikuchi.

In Trends in Biotechnology 14 (1996) 451 - 452 Robinson, C.J. et al. describe the intraventricular implantation of alginate microspheres as a method for the local application of NGF or NGF-secreting cells. An application for hedgehog proteins is, however, not described.

In J.Cell.Physiol. 152 (1992) 422 - 429 Downs, E.C. et al describe the use of calcium alginate spheres as a delivery system for angiogenesis factors. However, the use of this method or this delivery system for hedgehog proteins is not described.

In Biotechnol. Bioeng. 31 (1988) 607 - 612 Crey, C.J. and J. Dowsett describe the use of calcium/zinc alginate spheres as a delivery system for insulin. However, an application of this process to produce delivery systems for hedgehog proteins is not disclosed.

It is known from Yang et al., Development 124 (1997) 4393-4404 that high local hedgehog concentrations must prevail over a period of at least 16 h at the site of action in the body for a pharmaceutically effective in vivo activity. The carrier system for this described by Yang et al. i.e. the hedgehog-loaded chromatography medium affigel CM, the Ni agarose described by Marti et al., in Nature 375 (1995) 322-325 or the Affigel blue used by Lopez-Martinez et al., in Curr.Biol. 5 (1995) 791-796 or the heparin agarose particles that they used are less suitable for a pharmaceutical application since they are immunogenic and can cause inflammatory reactions.

The inventors found that the biocompatible and biodegradable carrier collagen described by Kinto et al. for hh-expressing cells is also unsuitable for an optimal local pharmaceutical application of hedgehog proteins. It was found that when the collagen carriers are loaded with hedgehog proteins under physiological conditions (pH ca. 7 and in weak acids (up to pH 4.5)) the majority of the applied hedgehog protein is released from the matrix within minutes. When the loading is carried out under acidic conditions (below pH 4.5) a large amount of the applied hedgehog protein is denatured and bound irreversibly to the carrier.

A pharmaceutical composition of a hedgehog protein is described in EP-A 98101893.0 which is characterized in that the hedgehog protein is bound to a hydrophilic carrier which is biocompatible wherein the carrier is a polymer which binds the hedgehog protein as a negatively-charged carrier due to ionic interactions, the hedgehog protein is not denatured during binding to the carrier, it contains at least 0.1 to 2 negatively-charged residues per monomer under neutral conditions, the charge is in the form of acidic groups, it has an average molecular weight of at least 50,000 Da and contains no agarose.

The object of the invention is to provide a pharmaceutical composition of a hydrophobically modified hedgehog protein containing a biocompatible carrier, wherein the carrier binds the hedgehog protein as an active folded structure and can release it locally in vivo in an active form in a delayed manner. Such formulations are particularly suitable for the repair of bone and cartilage defects and can also be used to repair neuronal defects or for a systemic delivery.

The object is achieved by a pharmaceutical composition of a hydrophobically modified hedgehog protein which is characterized in that this composition contains hydrophobically modified hedgehog proteins and a biodegradable protein or a proteolytic degradation product thereof as a carrier.

It has surprisingly turned out that hydrophobically modified hedgehog proteins can be released in vivo from such a carrier in a reversible, active and delayed manner without causing immunogenic and/or inflammatory reactions in vivo. The carrier can be a solid carrier such as a sponge or an injectable carrier such as a gel. The preferred biodegradable carrier is collagen or the hydrolysis product gelatin. Other fibrous carrier proteins such as fibrin or elastin are also suitable and can be used as intact protein fibres, as solubilized protein or as partially hydrolysed protein.

Collagen is understood according to the invention as preferably soluble collagen, insoluble collagen (preferably cross-linked), atelo-collagen or gelatin. According to the invention the collagen can be present as a solid matrix (cross-linked or non-cross-linked lyophilisate or precipitate, as fibres, as a dispersion or as a gel). Recombinantly produced collagen as well as type I or type II collagen and mixtures thereof are particularly suitable. Collagen fibres can for example be prepared according to the British Patent No. 1204438. Soluble collagen can for example be prepared by processes as described in the British Patents No. 990276 and 1184502. The product which is prepared by these methods can be referred to as a microgel and contains a mixture of collagen in various forms of aggregation. Hydrolysed collagen can preferably be obtained by treating collagen with trypsin. This results in a polydisperse mixture of polypeptides with a molecular weight in the range between about 5000 and 70000. The collagen is preferably firstly denatured for example by heat treatment before treatment with trypsin.

The biodegradable carrier, preferably collagen, is preferably used as a sponge such as for example the collagen sponge Helistat™ from the Integra Life Sciences Company, USA.

A collagen is preferably used as the carrier matrix, particularly preferably as a soluble or insoluble carrier matrix. The carrier matrix is particularly preferably composed of a combination of the biodegradable carrier and an anionic polysaccharide, such as preferably hyaluronic acid (as well as chemically cross-

linked forms thereof), chondroitin sulfate, polyvinyl sulfate, keratan sulfate, dextran sulfate, pectin, carrageenan and other hydrocolloids, sulfated alginate, dermatan sulfate, alginate, preferably calcium alginate or complexes of protein and polysaccharides such as those described for example in the US patent No. 4,614,794 (Fibracol™, Johnson and Johnson, USA) in which the percentage by weight of the charged polysaccharides is 10-50 %. An insoluble matrix in the sense of the invention means that the matrix does not substantially decompose or significantly dissolve in a neutral buffer solution in vitro within 10-20 hours at room temperature. In this connection it is preferred that the carrier used according to the invention contains less than 50 %, preferably less than 20 % and particularly preferably essentially no amount of a neutral polysaccharide. A combination of collagen with hyaluronic acid with a molecular weight of at least 10^6 Dalton, in particular with a molecular weight of 4×10^6 Dalton is particularly suitable.

A delayed release is understood according to the invention as a release of the hedgehog protein in a pharmacologically effective dose over a defined period of at least 14 hours. A pharmacologically effect is understood as a neurological effect on nerve cells, a chondrogenesis and/or chondroinduction and preferably osteogenesis and/or osteoinduction as described in Kinto et al., FEBS Letters, 404 (1997) 319-323 for bone induction, by Miao et al. in J.Neurosci. 17 (1997) 5891-5899 for the effect on nerve cells and by Stott et al. in J.Cell Sci. 110 (1997) 2691-2701 for cartilage cell induction.

An enzymatically degradable carrier is preferably used as the carrier which is degraded by enzymes (e.g. proteases) secreted from the cells on which the local in vivo application has occurred. However, the half life of the carrier should be at least 12 h but can be several weeks. If the carrier is composed of a polysaccharide, this carrier is preferably degraded by glycosidases and by hydrolases that are present in and secreted by the cell. However, such a biodegradability of the carrier is not necessary in every case8 If the release is carried out to treat osteoporosis or neuronal diseases, a biodegradability is unnecessary. However, such carriers are preferably poorly soluble under physiological conditions and are therefore absorbed by the body over a long period (several weeks to months).

Solutions of hedgehog proteins at high concentrations are required to produce carrier matrices that are coated with hedgehog proteins in such a manner that they exhibit an adequate pharmaceutical efficacy when applied locally. It has turned out

that pharmaceutically suitable carriers coated with hedgehog protein should preferably contain a concentration of the hedgehog protein of 1 - 20 mg/ml carrier and more. Carriers are particularly advantageous which contain hedgehog proteins at a concentration of 10 mg/ml carrier (solution volume, gel volume or sponge volume) or more. Hedgehog proteins are inherently poorly soluble. A further subject matter of the invention is a process for the production of a carrier matrix coated with hedgehog protein which is characterized in that the carrier matrix is incubated with a hedgehog protein solution at a concentration of 3 mg/ml which contains arginine or argininium ions and the carrier matrix coated in this manner is isolated.

Such solutions are suitable for producing carrier matrices which contain hedgehog proteins in pharmaceutically effective concentrations and are suitable for pharmaceutical applications. Hence a further subject matter of the invention is a collagen carrier which contains 3 mg hydrophobically modified hedgehog protein or more, preferably 10 mg or more per ml carrier matrix and arginine or argininium ions (preferably argininium sulfate). The concentration of arginine is

test). In this method a mouse fibroblast cell line is cultured in a medium which contains foetal calf serum. Subsequently sterile filtered sample is added, the cells are lysed after ca. 5 days and alkaline phosphatase is determined in the cell lysate by means of the cleavage of a chromogenic substrate (pNP, p-nitrophenol) (J. Asahina, Exp. Cell. Res. 222 (1996) 38 - 47 and T. Nakamura (1997)).

A hydrophobically modified (lipophilized) hedgehog protein is understood by the invention as a lipophilized secreted signal protein which is responsible for the formation of numerous structures in embryogenesis. Sonic, indian or desert hh are particularly preferably used (Fietz M. et al., Development (Suppl.) (1994) 43-51). The processed form (N-terminal mature domain) of sonic hh protein described in EMBL data bank under the No. L38518 is preferably used. Proteins of the hedgehog family exhibit a pronounced homology in their amino acid sequence which is why it is also preferable to express those nucleic acids which code for hedgehog proteins

which are 80 % or more homologous with the above-mentioned sequence of sonic hedgehog protein.

Such a lipophilization is preferably achieved by chemical modification. Such a hedgehog conjugate preferably contains an additional polypeptide that is covalently bound (preferably at the C-terminus and/or N-terminus) and is composed of 10 - 30 preferably hydrophobic amino acids and/or those amino acids which form transmembrane helices. The additional polypeptide particularly preferably contains 2 - 12 lysines and/or arginines but no polyhistidine part that would be suitable for purifying the conjugate on a Ni chelate column. It is also preferable to covalently bind (preferably at the C-terminus and/or N-terminus) 1 - 4 aliphatic, saturated or unsaturated hydrocarbon residues with a chain length of 10 - 24 C atoms or steroids with a lipophilic (hydrophobic) action. Furthermore it is preferred to covalently couple hydrophobic thio compounds, such as in particular thiocholesterol and thioalkanes, thioalkenes, to hh proteins via a disulfide bridge formed oxidatively (preferably at the C-terminus and/or N-terminus and in this case on the N-terminal cysteine).

The protein is hydrophobized by such lipophilizing residues which improves its interaction with lipid membranes of eukaryotic cells, in particular of mammalian cells.

Consequently a lipophilized protein according to the invention is understood as a hydrophobized protein which has an increased surface hydrophobicity compared to an unmodified protein which increases its affinity for apolar molecules or amphiphiles. The increase in the degree of lipophilicity of the protein can be measured by the degree of integration in a lipid layer as described for example by Haque, Z. et al., J.Agric.Food Chem. 30 (1982), 481. Methods for the hydrophobic (lipophilizing) modification of proteins are for example described by Haque, Z. et al., J.Agric.Food Chem. 31 (1983) 1225-1230; Webb, R.J. et al., Biochemistry 37 (1998) 673-679; Hancock, J.F., Cell 63 (1990) 133-139; A Practical guide to membrane protein purification, Ed. G.v. Jagow, Hermann Schägger (1994), (chapter 16, pages 535-554).

Such hydrophobically modified hedgehog proteins are described for example in the European Patent Applications No. 98116733.1 and 98107911.4.

The human sonic hedgehog precursor protein is composed of the amino acids 1 - 462 of the sequence described in the EMBL databank under No. L38518. The amino acids 1 - 23 represent the signal peptide, the amino acids 24 - 197 represent the mature signal domain, the amino acids 32 - 197 represent the signal domain shortened by eight amino acids and the amino acids 198 - 462 represent the autoprocessing C-terminal domain after autoproteolytic cleavage.

The pharmaceutical composition according to the invention contains a pharmacologically effective dose of the hh protein and can be administered locally. It is preferable to use the proteins according to the invention in combination with other proteins of the hedgehog family or bone growth factors such as bone morphogenetic proteins (BMPs) (Wozney et al., Cell.Mol.Biol. of Bone, Bone Morphogenetic Proteins and their Gene Expression (1993) Academic Press Inc., 131-167) or parathyroid hormones (Karablis et al., Genes and Development 8 (1994) 277-289) or insulin-like growth factors (IGF-I or II) or transforming growth factors (TGF-β).

The pharmaceutical composition is produced by incubating the lipophilized hedgehog protein with the carrier or with the carrier containing collagen at a pH value of 4.5 or more. The incubation is preferably carried out at a neutral pH value (pH 6-8) and preferably in a buffered solution. At pH 4.5 or higher pH values, the lipophilized hedgehog protein binds to the carrier by hydrophobic interactions. If the carrier matrix additionally contains a hydrophilic carrier which can bind proteins by means of ionic interactions (for example an anionic polysaccharide), the hedgehog protein can then also interact with this part of the carrier by means of ionic interactions. For this reason the ratio of collagen to the hydrophilic carrier moiety can vary over a wide range. However, it is preferable that the ratio of collagen moiety to hydrophilic carrier moiety is 1.5:1 or more.

Furthermore it is preferable for the production of the pharmaceutical composition to add auxiliary substances such as a sugar (mannitol, lactose, glucose, sucrose, trehalose, preferably 20-100 mg/ml) or amino acids such as glycine or arginine, oxidation inhibitors such as methionine as well as antioxidants such as EDTA, citrate, polyethylene glycol (1 - 10 % by weight), detergents, preferably non-ionic detergents (preferably 0.005 - 1 % by weight) such as polysorbates (Tween®20 or Tween®80) or polyoxyethylenes, anti-inflammatory agents, local anaesthetics, antibiotics and/or stabilizers such as lipids, fatty acids and glycerol.

In a further preferred embodiment a pharmaceutical composition of the hedgehog protein according to the invention containing suramin is preferred and this can be used advantageously.

The pharmaceutical composition can contain additional pharmaceutical auxiliary substances.

In a preferred embodiment the pharmaceutical composition contains the lipophilized hedgehog protein at a concentration of 0.1 - 100 mg/ml.

In a preferred embodiment the pharmaceutical composition additionally contains a pharmaceutically acceptable buffer which is biocompatible, preferably in the range between pH 4 and pH 10, particularly preferably in the range between pH 6 and 8. The pH value of the pharmaceutical composition should be advantageously greater than pH 4 in order to prevent denaturation and detachment of the zinc complexed in the hedgehog protein. The concentration of the buffer is preferably 1-500 mmol/l, in particular 5-150 mmol/l and particularly preferably 10-100 mmol/l. In a suitable embodiment 300 mmol/l potassium phosphate buffer, pH 6.0 or 300 mmol/l arginine chloride, 10 mM potassium phosphate, pH 6.0 is used as a buffer.

The following examples, publications and the figures further elucidate the invention, the protective scope of which results from the patent claims. The described methods are to be understood as examples which still describe the subject matter of the invention even after modifications.

Description of the Figures:

Fig. 1: In vitro release of hydrophobically modified shh from a collagen matrix (Helistat™).

Fig. 2: In vitro release of dipalmityl-shh from a collagen/alginate matrix (Fibracol™).

Examples

Example 1

In vitro release of hydrophobically modified hedgehog proteins from a collagen matrix (Helistat™)

0.1 ml aliquots of solutions of variously modified hedgehog proteins (palmityl-shh = 1xC16-shh, dipalmityl-shh = 2xC16-shh or cholesteryl-shh = Chol-shh) are applied dropwise to collagen sponges (Helistat™, Integra Life Sciences, USA) with dimensions of 10 x 10 x 3 mm. The loaded carriers are frozen (-70°C), lyophilized and analysed. For this the sponges are incubated at 37°C in a suitable volume of PBS. The amount of released hh is determined by means of rp-HPLC (see Fig. 1).

The analysis of the in vitro release kinetics shows that Shh is released in a delayed manner or in small amounts from the collagen matrix over a period of 20 h (Fig. 1).

It can be seen that in vitro only ca. 20 % of the loaded 2xC16-shh is released under the stated conditions. The remaining 80 % of the applied protein can be released in vivo by biodegradation of the carrier. This situation can be simulated in vitro by an enzymatic degradation of the collagen carrier with recombinant collagenase. For this a collagen sponge loaded with 2xC16-shh which had been incubated for 14 h under release conditions (see above) was transferred into 490 µl buffer (50 mM HEPES-NaOH, 800 mM NaCl, 0.1 % Tween, pH 7.4) and 10 µl recombinant collagenase I (1 mg/ml, in 10 mM Tris-Cl, 1 mM $CaCl_2$, pH 7.4) was added. After incubation for 2 h at 37°C the released hedgehog protein is analysed by means of reversed phase HPLC and in an in vitro activity test (induction of alkaline phosphatase in C3H10T1/2 cells). It is clear from this analysis that the non-released fraction of 2xC16-shh (see above) that remains in the collagen carrier under release conditions can be released by means of collagenase digestion and its activity is not lost in this process (ca. 80 % recovery).

Example 2:

In vitro release of hydrophobically modified hedgehog protein (dipalmityl-shh) from a collagen-alginate matrix (Fibracol™)

0.05 ml aliquots of a solution of dipalmityl-shh (1 mg/ml) are added dropwise to collagen-alginate sponges (Fibracol™; Johnson & Johnson, USA) of approximately 10 x 10 x 3 mm size. The loaded carriers are frozen (-70°C), lyophilized and analysed. For this the sponges are incubated at 37°C in a suitable volume of PBS. The amount of released 2xC16-shh is determined by means of rp-HPLC (see Fig. 2).

It can be seen that in vitro only ca. 10 % of the loaded 2xC16 is released under the stated conditions. The remaining 90 % of the applied protein can be released in vivo by biodegradation of the carrier. This situation can be simulated in vitro by a partial enzymatic degradation of the carrier with recombinant collagenase. For this the carriers loaded with 2xC16-shh which had been incubated under release conditions (see above) were transferred into 490 µl buffer (50 mM HEPES-NaOH, 800 mM NaCl, 0.1 % Tween, pH 7.4) and 10 µl recombinant collagenase I (1 mg/ml, in 10 mM Tris-Cl, 1 mM $CaCl_2$, pH 7.4) was added. After incubation for 2 h at 37°C the released hedgehog protein is analysed after centrifugation by means of reversed phase HPLC. It is clear from this analysis that the non-released fraction of 2xC16-shh that remains in the collagen-alginate carrier under release conditions can be released by means of collagenase digestion (ca. 80 % recovery; Fig. 2).

List of References

A Practical guide to membrane protein purification, Ed. G.v. Jagow, Hermann Schägger (1994), chapter 16, pages 535 - 554

Asahina, J., Exp. Cell. Res. 222 (1996) 38-47

Bitgood, M.J. et al., Curr. Biol. 6 (1996) 296

British Patent No. 1184502

British Patent No. 1204438

British Patent No. 990276

Chiang, C. et al., Nature 83 (1996) 407

Crey, C.J. et al., Biotechnol.Bioeng. 31 (1988) 607-612

Downs, E.C. et al., J.Cellular Physiology 152 (1992) 422-429

EP-A 98 101 893.0

European Patent Application No. 98107911.4

European Patent Application No. 98116733.1

Fietz, M. et al., Development (Suppl) (1994) 43-51

Hancock, J.F., Cell 63 (1990) 133-139

Haque, Z.et al., J. Agric.Food Chem. 30 (1982) 481

Haque, Z. et al., J. Agric.Food Chem. 31 (1983) 1225-1230

Hynes, M. et al., Neuron 15 (1995) 35-44

Karablis et al., Genes and Development 8 (1994) 277-289

Kikuchi, A. et al., J. Controlled Release 47 (1997) 21-29

Kinto et al., FEBS Letters, 404 (1997) 319-323

Lai, C.J. et al., Development 121 (1995) 2349

Lopez-Martinez et al. in Curr.Biol. 5 (1995) 791-796

Marti et al., Nature 375 (1995) 322-325

Miao et al., J. Neurosci. 17 (1997) 5891-5899

Nakamura, T. et al., Biochem. Biophys. Res. Comm. 237 (1997) 465-469

Perrimon, N., Cell 80 (1995) 517-520

Porter, J.A. et al., Science 274 (1996) 255-259

Robinson, C.J. et al., Trends in Biotechnology 14 (1996) 451-452

Smith, J.C., Cell 76 (1994) 193-196

Stott et al., J. Cell Sci. 110 (1997) 2691-2701

US-Patent 4,614,794

Vortkamp, A. et al., Science 273 (1996) 613

Webb, R.J. et al., Biochemistry 37 (1998) 673-679

WO 90/08551

Wozney et al., Cell. Mol. Biol. of Bone, Bone Morphogenetic Proteins and their Gene Expression, (1993) Academic Press Inc. 131-167

Yang et al., Development 124 (1997) 4393-4404

replacement sheet

Claims

1. Pharmaceutical composition containing a hydrophobically modified hedgehog protein and a biodegradable protein as a carrier.

2. Pharmaceutical composition as claimed in claim 1, containing soluble collagen as a carrier.

3. Pharmaceutical composition as claimed in claim 1, containing insoluble, cross-linked collagen.

4. Pharmaceutical composition as claimed in the claims 1 - 3, containing a hyaluronic acid or alginate.

5. Pharmaceutical composition as claimed in claims 1 -4, containing a hedgehog protein at a concentration of 0.1 - 100 mg/ml.

6. Pharmaceutical composition as claimed in claims 1 -5, wherein the composition is buffered in a range between pH 4.5 and 10.

7. Pharmaceutical composition as claimed in claims 1 - 6, containing arginine or argininium ions.

8. Process for the production of a pharmaceutical composition, wherein a hydrophobically modified hedgehog protein is combined in a therapeutically effective amount with a biodegradable protein as a carrier.

9. Process for the delayed release of a hydrophobically modified hedgehog protein in the human body, wherein the said hedgehog protein is applied locally in the human body in a pharmaceutical composition as claimed in claims 1 - 7.

10. Process for the production of an insoluble, biodegradable protein carrier matrix which contains a hydrophobically modified hedgehog protein, wherein the carrier matrix is incubated with a solution containing the said hedgehog protein at a concentration of 3 mg/ml or more and arginine or

argininium ions at a concentration of 10 mmol/l or more and the carrier matrix coated in this manner is isolated.

ersatzblatt (regel 26)

Fig. 1

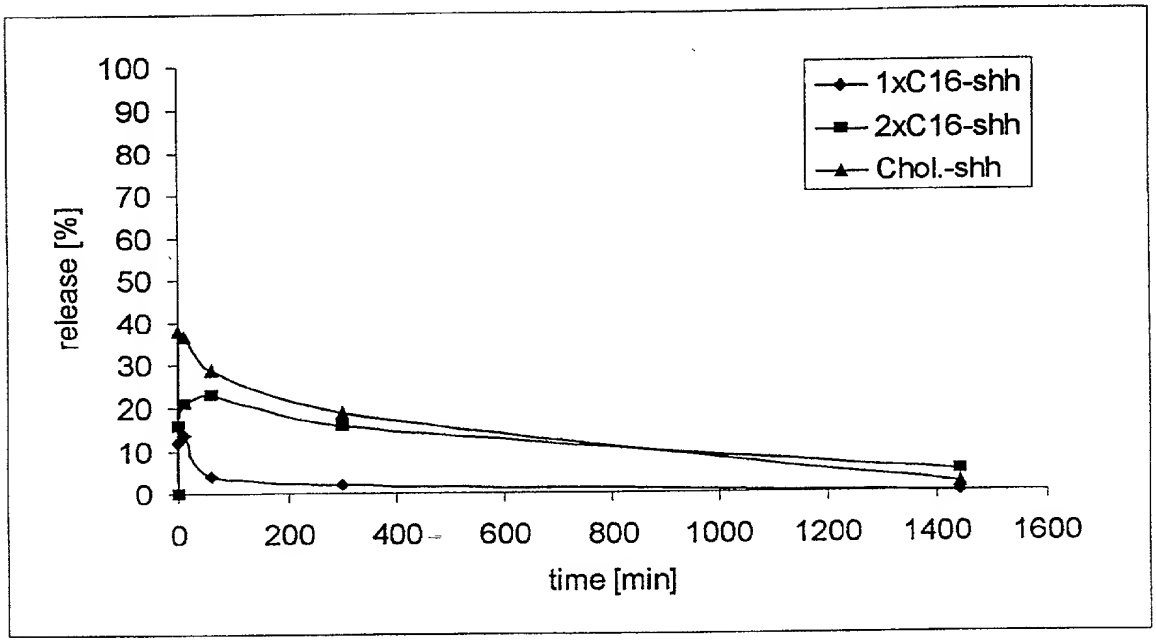
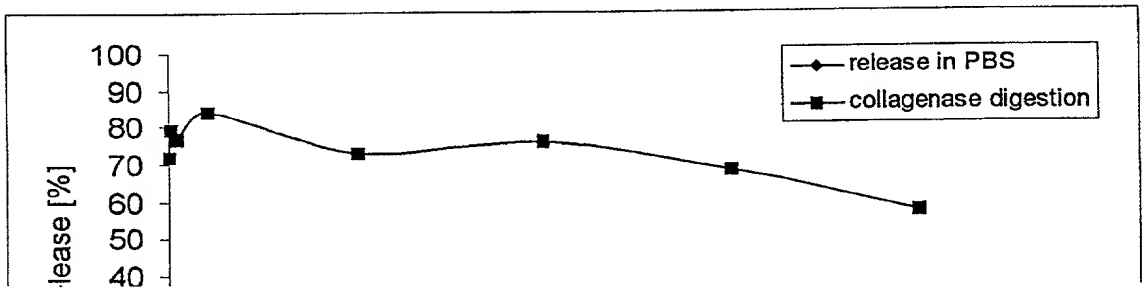

100
90
80
70
60
50
40
30
20
10
0
release [%]
0
200
400
600
800
1000
1200
1400
1600
time [min]
1xC16-shh
2xC16-shh
Chol.-shh

Fig. 2


100
90
80
70
60
50
40
release [%]
release in PBS
collagenase digestion

Docket No.
CIBT-P01-097

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PHARMACEUTICAL COMPOSITIONS OF HYDROPHOBICALLY MODIFIED HEDGEHOG PROTEINS AND THEIR USE

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **July 25, 2001** as United States Application No. or PCT International Application Number **09/890,053**

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
99101643.7 (Number)	**EP** (Country)	**4 February 1999** (Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/EP00/00847	**3 February 2000**	**Pending**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Madeline F. Baer, Reg. No. 36,437
Steven J. Baughman, Reg. No 47,414
Johnny Y. Chen, Reg. No. 46,614
Michael P. Doyle, Reg. No. P-49,052
Gregory G. Glover, Reg. No. 34,173
William G. Gosz, Reg. No. 27,787
Patricia Granahan, Reg. No. 32,227
David P. Halstead, Reg.No. 44,735
Daniel Hansburg, Reg. No. 36,156
Edward J. Kelly, Reg. No. 38,936
Paul E. Lewkowicz, Reg. No. 44,870
Robert A. Mazzarese, Reg. No. 42,852
Sanjay Sitlani, Reg. No. 47,558
Wolfgang Stutius, Reg. No. 40,256
Matthew P. Vincent, Reg. No. 36,709

Send Correspondence to: **Matthew P. Vincent**
Patent Group
Ropes & Gray
One International Place, Boston, MA 02110

Direct Telephone Calls to: *(name and telephone number)*
Matthew P. Vincent (617) 951-7000

Full name of sole or first inventor **PAPADIMITRIOU, Apollon**
Sole or first inventor's signature Apollon Papadimitriou Date 8/29/01
Residence **Bachstrabe 38a, D-83673 Bichl, DE** DEX
Citizenship **DE**
Post Office Address **Same as above**

Full name of second inventor, if any **LANG, Kurt**
Second inventor's signature Kurt Lang Date 9/19/01
Residence **Langoner Strabe 10, D-82377, Penzberg, DE** DEX
Citizenship **DE**
Post Office Address **Same as above**